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(54) Title: AN ENZYME WITH PECTIN ESTERASE ACTIVITY

(57) Abstract

The present invention relates to an enzyme with pectin esterase activity, a DNA construct encoding the enzyme with pectin esterase activity, a method of producing the enzyme, an enzyme composition comprising said enzyme with pectin esterase activity, and the use of said enzyme and enzyme composition for a number of industrial applications.

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TITLE: An enzyme with pectin esterase activity

FIELD OF INVENTION

5 The present invention relates to an enzyme with pectin esterase activity, a DNA construct encoding the enzyme with pectin esterase activity, a method of producing the enzyme, an enzyme composition comprising said enzyme with pectin esterase activity, and the use of said enzyme and enzyme composition
10 for a number of industrial applications.

BACKGROUND OF THE INVENTION

15 Pectin polymers are important constituents of plant primary cell walls. They are composed of chains of 1,4-linked α -D-galacturonic acid and methylated derivatives thereof. The use of pectin-degrading enzymes such as polygalacturonase, pectin methylesterase, pectin lyase or pectate lyase is important for
20 the food industry, primarily in fruit and vegetable processing such as fruit juice production or wine making, where their ability to catalyse the degradation of the backbone of the pectin polymer is utilised.

25 For many purposes, it would be desirable to provide each of the pectin degrading enzymes present in, for instance, commercial preparations containing a number of different pectin degrading enzymes (an example of such a preparation is Pectinex Ultra SP[®], prepared from *Aspergillus aculeatus*, available from Novo Nordisk A/S) in a form free from other
30 components. In this way, it would be possible to produce enzyme preparations adapted to specific purposes, such preparations either containing a single pectin degrading enzyme or arbitrary combinations thereof. To serve this end, it is convenient to provide single-component pectin degrading
35 enzymes by recombinant DNA techniques.

Pectin methylesterase (EC 3.1.1.11) catalyses the removal of methanol from pectin, resulting in the formation of pectic acid (polygalacturonic acid).

5 Pectin methylesterases are produced by a number of microorganisms and have also been found to be present in leaves, roots, stalks and fruits of many higher plants. Microbial pectin methylesterases have been cloned by Khanh et al. (1990) "Nucleotide and derived amino acid sequence of a pectinesterase cDNA isolated from *Aspergillus niger* strain RH5344", Nucleic Acids Res. 18:4262; Khanh et al. (1991) "Characterization and expression of a genomic pectin methyl esterase-encoding gene in *Aspergillus niger*", Gene 106:71-77; Spok et al. (1991) "Molecular cloning and sequencing of a pectinesterase gene from *Pseudomonas solanacearum*", J. Gen. Microbiol. 137:131-140; Plastow (1988) "Molecular cloning and nucleotide sequence of the pectin methyl esterase gene of *Erwinia chrysanthemi* B374", Mol. Microbiol. 2:247-254; Laurent et al. (1993) "Characterization and overexpression of the pem gene encoding pectin methylesterase of *Erwinia chrysanthemi* strain 3937", Gene 131:17-25; Tierny et al. (1994) "Molecular cloning and expression in *Escherichia coli* of genes encoding pectate lyase and pectin methylesterase activities from *Bacteroides thetaiotaomicron*", J. Appl. Bacteriol. 76:592-602.

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WO 94/25575 describes the cloning of a pectin methylesterase from *Aspergillus aculeatus*.

SUMMARY OF THE INVENTION

30 According to the present invention, the inventors have now succeeded in isolating and characterizing a DNA sequence, from a Basidiomycota fungus, which encodes an enzyme exhibiting pectin methylesterase also known as pectin esterase activity, thereby making it possible to prepare a mono-component pectin esterase composition.

Accordingly, in a first aspect the invention relates to a DNA construct comprising a DNA sequence encoding an enzyme exhibiting pectin esterase activity, which DNA sequence comprises

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a) the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *E. coli* DSM 10357, or

10 b) an analogue of the DNA sequence defined in a) which

i) is at least 60% homologous with the DNA sequence defined in a), or

15 ii) hybridizes with the DNA sequence shown in positions 4-933 in SEQ ID NO 1 at low stringency, or

20 iii) encodes a polypeptide which is at least 50% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence defined in a), or

25 iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified pectin esterase encoded by the DNA sequence defined in a).

30 The full length cDNA sequence encoding a pectin esterase has been derived from a strain of the filamentous fungus *Meripilus giganteus* and has been cloned into plasmid pYES 2.0 present in the *Escherichia coli* strain DSM No. 10357.

35 Said pectin esterase encoding DNA sequence harboured in *E. coli* DSM 10357 is believed to have the same sequence as that presented in SEQ ID NO 1. Accordingly, whenever reference is

made to the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 10357 such reference is also intended to include the pectin esterase encoding part of the DNA sequence presented in SEQ ID NO 1.

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Accordingly, the terms "the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 10357" and "the pectin esterase encoding part of the DNA sequence presented in SEQ ID NO 1" may be used

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In further aspects the invention provides an expression vector harbouring the DNA construct of the invention, a cell comprising said DNA construct or said expression vector and a method of producing an enzyme exhibiting pectin esterase activity, which method comprises culturing said cell under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

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In a still further aspect the invention provides an enzyme exhibiting pectin esterase activity, which enzyme,

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(a) is encoded by a DNA construct of the invention; or
(b) is produced by the method of the invention; and/or

30

(c) is immunologically reactive with an antibody raised against a purified pectin esterase encoded by the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *E. coli* DSM 10357. In a preferred embodiment the enzyme has the deduced amino acid sequence SEQ ID NO 2.

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In a still further aspect, the present invention provides an enzyme composition useful for the degradation or modification of plant material or components thereof, said composition being enriched with an enzyme exhibiting pectin esterase activity as described above.

In a still further aspect, the present invention relates to the use of an enzyme or an enzyme composition of the invention for various industrial applications.

5

Finally the invention relates to an isolated substantially pure biological culture of the *E. coli* strain DSM No. 10357 harbouring a pectin esterase encoding DNA sequence (the pectin esterase encoding part of the DNA sequence cloned into plasmid 10 pYES 2.0 present in *E. coli* DSM 10357) derived from a strain of the filamentous fungus *Meripilus giganteus*, or any mutant of said *E. coli* strain having retained the pectin esterase encoding capability; and to an isolated substantially pure biological culture of the filamentous fungus *Meripilus* 15 *giganteus* CBS No. 521.95, from which the DNA sequence presented as SEQ ID No. 1 has been derived.

DETAILED DESCRIPTION OF THE INVENTION

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DNA Constructs

The present invention provides a DNA construct comprising a DNA sequence encoding an enzyme exhibiting pectin esterase activity, which DNA sequence comprises

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a) the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *E. coli* DSM 10357, or

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b) an analogue of the DNA sequence defined in a) which

i) is at least 60% homologous with the DNA sequence defined in a), or

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ii) hybridizes with the DNA sequence shown in positions 4-933 in SEQ ID NO 1 at low stringency, or

iii) encodes a polypeptide which is at least 50% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence defined in a), or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified pectin esterase encoded by the DNA sequence defined in a).

As defined herein, a DNA sequence analogous to the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *E. coli* DSM 10357 is intended to indicate any DNA sequence encoding an enzyme exhibiting pectin esterase activity, which enzyme has one or more of the properties cited under (i)-(iv) above.

20 The analogous DNA sequence may be isolated from a strain of
the filamentous fungus *Meripilus giganteus* producing the
enzyme with pectin esterase activity, or another or related
organism and thus, e.g. be an allelic or species variant of
the pectin esterase encoding part of the DNA sequence cloned
into plasmid pYES 2.0 present in *E. coli* DSM 10357.

30 Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as the pectin esterase encoding part of SEQ ID No. 1, e.g be a sub-sequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the pectin esterase encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence.

When carrying out nucleotide substitutions, amino acid changes are preferably of a minor nature, that is conservative amino

acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

10 Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), 15 aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine). For a general description of nucleotide substitution, see e.g. Ford et al., (1991), Protein Expression and Purification 2, 95-107.

20 It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and 25 therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. e.g. Cunningham and Wells, (1989), Science 244, 1081-1085). In 30 the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (i.e. pectin esterase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be 35 determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (cf. e.g. de Vos et

al., (1992), Science 255, 306-312; Smith et al., (1992), J. Mol. Biol. 224, 899-904; Wlodaver et al., (1992), FEBS Lett. 309, 59-64).

5 The homology referred to in i) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package
10 (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP
15 creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, and even more preferably at
20 least 97%, with the pectin esterase encoding part of the DNA sequence shown in SEQ ID No. 1.

25 The hybridization referred to in (ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the pectin esterase enzyme under certain specified conditions which are described in detail in the Materials and Methods section hereinafter. The oligonucleotide probe to be used is the DNA sequence corresponding to the pectin esterase encoding part of the DNA
30 sequence shown in SEQ ID NO 1.

35 The homology referred to in iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August

1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the polypeptide encoded by an analogous DNA sequence exhibits a degree of identity preferably of at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, and especially at least 97% with the enzyme encoded by a DNA construct comprising the pectin esterase encoding part of the DNA sequence shown in SEQ ID No. 1; e.g. with the amino acid sequence SEQ ID NO 2.

The present invention is also directed to pectin esterase variants which have an amino acid sequence which differs by no more than three amino acids, preferably by no more than two amino acids, and more preferably by no more than one amino acid from the mature part of the amino acid sequence set forth in SEQ ID NO 2.

In connection with property iv) the immunological reactivity may be determined by the method described in the Materials and Methods section below.

The DNA sequence encoding a pectin esterase of the invention can be isolated from the strain *Escherichia coli* DSM 10357 using standard methods e.g. as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence encoding an enzyme exhibiting pectin esterase activity of the invention can also be isolated by any general method involving

- cloning, in suitable vectors, a cDNA library from any organism expected to produce the pectin esterase of interest,

transforming suitable yeast host cells with said vectors,
culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,
screening for positive clones by determining any pectin esterase activity of the enzyme produced by such clones, and
isolating the enzyme encoding DNA from such clones.

10 A general isolation method has been disclosed in WO 93/11249 or WO 94/14953, the contents of which are hereby incorporated by reference. A more detailed description of the screening method is given in Example 1 below.

15 Alternatively, the DNA encoding a pectin esterase of the invention may, in accordance with well-known procedures, conveniently be isolated from a suitable source, such as any of the below mentioned organisms, by use of synthetic 20 oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the pectin esterase encoding part of the nucleotide sequences presented as SEQ ID No. 1 or any suitable subsequence thereof, or the basis of the 25 amino acid sequence SEQ ID NO 2.

Microbial Sources

30 In a preferred embodiment, the DNA sequence encoding the pectin esterase is derived from a strain belonging to the *Polyporaceae* family, which according to the entrez browser NCBI taxonomy version 3,3, (updated 95.12.13) is a family within the order *Aphyllophorales*, which belongs to the class of *Hymenomycetes* under the *Basidiomycota*.

35 It is at present contemplated that a DNA sequence encoding an enzyme homologous to the enzyme of the invention, i.e. an

analogous DNA sequence, may be obtained from other microorganisms. For instance, the DNA sequence may be derived by similarly screening a cDNA library of another microorganism, such as a strain of *Aspergillus*, *Saccharomyces*,
5 *Bacteroides*, *Erwinia*, *Pseudomonas* or *Clostridium*.

An isolate of a strain of *Meripilus giganteus* from which a pectin esterase of the invention can be derived has been deposited by the inventors according to the Budapest Treaty on
10 the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Centraalbureau voor Schimmelcultures, P.O. Box 273, 3740 AG Baarn, The Netherlands (CBS).

15 Deposit date : 04.07.95
Depositor's ref. : NN006040
CBS designation : *Meripilus giganteus* CBS No. 521.95

20 The expression plasmid pYES 2.0 comprising the full length cDNA sequence encoding the pectin esterase of the invention has been transformed into a strain of the *E. coli* which was deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutsche Sammlung
25 von Mikroorganismen und Zellkulturen GmbH., Masheroder Weg 1b, D-38124 Raunschweig, Federal Republic of Germany, (DSM).

Deposit date : 06.12.95
Depositor's ref. : NN049144
30 DSM designation : *Escherichia coli* DSM 10357

Expression vectors

In another aspect, the invention provides a recombinant expression vector comprising the DNA construct of the
35 invention.

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector 5 may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced 10 into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the expression vector, the DNA sequence encoding the pectin 15 esterase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures 20 used to ligate the DNA sequences coding for the pectin esterase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., 1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY.

25

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. 30 Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral α -amylase, *Aspergillus niger* acid stable α -amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, 35 *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Host cells

In yet another aspect the invention provides a host cell comprising the DNA construct of the invention and/or the 5 recombinant expression vector of the invention.

Preferably, the host cell of the invention is a eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Trichoderma*, preferably *Trichoderma harzianum* or

10 *Trichoderma reesei*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*, or a species of *Fusarium*, in particular a strain of *Fusarium graminearum*, *Fusarium cerealis*. Fungal cells may be transformed by a 15 process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

20 The host cell may also be a yeast cell, e.g. a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp., such as 25 *Yarrowia lipolytica*, or

Kluyveromyces sp., such as *Kluyveromyces lactis*.

Method of producing pectin esterase

In a still further aspect, the present invention provides a 30 method of producing an enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

35

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in

question. The expressed pectin esterase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, 5 precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

10 Enzyme compositions

In a still further aspect, the present invention relates to an enzyme composition useful for the modification or degradation 15 of plant cell wall components, said composition being enriched in an enzyme exhibiting pectin esterase activity as described above.

20 The enzyme composition having been enriched with an enzyme of the invention may e.g. be an enzyme composition comprising multiple enzymatic activities, in particular an enzyme composition comprising multiple plant cell wall degrading enzymes such as Viscozym[®], Pectinex[®] or Pectinex Ultra SP[®] (all available from Novo Nordisk A/S). In this manner a boosting of the cell wall degrading ability of the enzyme 25 composition can be obtained.

25 In the present context, the term "enriched" is intended to indicate that the pectin esterase activity of the enzyme 30 composition has been increased, e.g. with an enrichment factor of 1.1, conveniently due to addition of an enzyme of the invention prepared by the method described above.

35 Alternatively, the enzyme composition enriched in an enzyme exhibiting pectin esterase activity may be one which comprises an enzyme of the invention as the major enzymatic component, e.g. a mono-component enzyme composition.

The enzyme composition may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. For instance, the enzyme composition may be in the form of a granulate or a microgranulate. The enzyme to be included in the composition may be stabilized in accordance with methods known in the art.

Examples are given below of preferred uses of the enzyme composition of the invention. The dosage of the enzyme composition of the invention and other conditions under which the composition is used may be determined on the basis of methods known in the art.

The enzyme composition according to the invention may be useful for at least one of the following purposes.

Degradation or modification of plant material:

The enzyme composition may advantageously be used for the treatment of pectin containing plant material, e.g. of vegetable or fruit origin, such as material obtained from soy beans, sugar beets or apples, so as to reduce the viscosity and thus improve the processing or appearance of the plant material in question. The viscosity reduction may be obtained by treating the pectin-containing plant material with an enzyme preparation of the invention under suitable conditions for full or partial degradation of the pectin-containing material.

The enzyme composition may be used for de-pectinization and viscosity reduction in vegetable or fruit juice, especially in apple or pear juice.

The enzyme preparation may be used in the treatment of mash from fruits and vegetables, for instance in the mash treatment of apples and pears for juice production, and in the mash treatment of grapes for wine production.

The enzyme composition may be used in the production of citrus juice, e.g. for partial or complete degradation of the pulp present in the juice after pressing.

5 For the above uses it is preferred that the enzyme composition in addition to pectin esterase comprises a polygalacturonase containing enzyme preparation.

10 By use of an enzyme preparation of the invention it is possible to regulate the consistency and appearance of processed fruit or vegetables. Thus, the consistency and appearance have been found to be a product of the actual combination of enzymes used for the processing, i.e. the 15 nature of the enzymes (especially pectin degrading enzyme(s)) with which the pectin methylesterase of the invention is combined.

20 Examples of products with specific properties which may be produced by use of an enzyme preparation of the invention include clear juice from apples, pears or berries, cloud stable juice from apples, pears, berries, citrus, or tomatoes, and purees from carrots and tomatoes.

25 From the foregoing disclosure it will be apparent that the pectinesterase of the invention may be produced as a single component essentially free from other enzyme activities such as polygalacturonase and/or pectin lyase activity normally found to be present in commercially available pectinesterase containing pectinolytic preparations.

30 On this basis the use of the pectin esterase of the invention is especially advantageous for purposes in which the action of such other enzyme activities is undesirable.

35 Examples of such purposes include the use of the pectin methylesterase for full or partial demethylation of pectin in processed or non-processed fruits and vegetables.

The partial demethylation is, e.g., important when an improved firmness of fruits or vegetables is desirable. Thus, firmness is often reduced during processing (e.g. canning and pasteurization). By use of a controlled amount of a pectin esterase of the invention a partial demethylation of pectin present in the fruits and vegetables may be obtained and the resulting partially demethylated pectin may crosslink with, e.g., divalent ions such as calcium, whereby a more firm fruits or vegetables may be formed. Accordingly, the pectin esterase of the invention may be used for improving the firmness of, e.g., red and green pepper, beans, peas and sliced fruits such as pears and apples.

Infusion of the enzyme can be performed e.g. unassisted by immersion or assisted by vacuum treatment.

Another example of the purposes is demethylation of pectin, e.g. from citrus, apple, sunflower and/or sugar beet.

Furthermore, the pectin esterase can be used to obtain an *in situ* viscosity increase or gel formation in various vegetable or fruit based products.

The pectin esterase of the invention can alone or together with other enzymes be used to improve the digestibility of pectin containing animal feed, e.g. feed prepared from soya beans, sugar beets or rape seeds. For this purpose, an enzyme composition of the invention is added to the feed.

The pectin esterase activity can together with other enzymes be used to produce monogalacturonic acid or galacturonic acid containing oligosaccharides from pectin-containing material such as sugar beet pulp in accordance with well-known methods. Monogalacturonic acid may be used for production of galactaric acid or for production of fatty acid and fatty alcohol esters and/or ethers of galacturonic acid. Galacturonic containing oligosaccharides may be used as additives for human food or

animal feed.

Furthermore, the pectin esterase can in combination with other enzymes be used for the removal of pectic substances from 5 plant fibres, which removal is essential, e.g. in the production of textile fibres or other cellulosic materials. For this purpose plant fibre material is treated with a suitable amount of the pectin esterase of the invention under 10 suitable conditions for obtaining full or partial degradation of pectic substances associated with the plant fibre material.

Mode of Action Block-wise vs Random Cleavage:

15 The distribution of the carboxyl groups in pectin is of importance for the functional properties of the pectin.

Several methods to determine the distribution of the free carboxyl groups on pectin have been described (Grasdalen, H. et al, *Carbohydrate Res.*, **289**, 105 (1995)). With the aim of 20 characterizing the mode of action of a pectin esterase the distribution of the acid groups is determined by a method described by Mort et al. (Mort, A.J. et al, *Carbohydr. Res.*, **247**, 21 (1993)). The esterified galacturonic acids are converted to galactose by reduction with sodium borohydride. 25 Subsequently the glycosidic linkages of the resulting galactose residues are cleaved selectively by HF solvolysis. This leads to the production of oligomers: (Gal A)_n-Gal. These oligomers represent the contiguous stretches of Gal A residues between methyl-esterified residues in the pectin. The cloned esterase of the invention was compared to an orange 30 esterase and to alkaline treatment. By high-performance anion exchange chromatography oligomers of six galacturonic acid residues were separated and quantified. From the distribution of these oligomers it can be determined whether the acid groups in the pectin are randomly or blockwise distributed.

35 Grasdalen et al, 1995 describes NMR spectroscopy as a useful method to obtain quantitative information on the composition

and the sequential structure of pectin. Contrary to the method defined by Mort et al 1993 NMR spectroscopy represents a direct approach to verify sequential structure, and only a moderate degree of polymer degradation is needed.

5

Without being bound to any theory, it is presently believed that the improved performance of the pectin esterase of the invention for a number of industrial applications such as in gelatination of jam (see Example 4 herein), is due to the 10 mode of action of the enzyme of the invention, preferably providing a block-wise distribution of the acid groups in the pectin.

15 The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

MATERIALS AND METHODS

20 **Deposited organisms:**

Meripilus giganteus CBS 521.95 comprises the pectin esterase encoding DNA sequence of the invention.

25 *Escherichia coli* DSM 10357 containing the plasmid comprising the full length cDNA sequence, coding for the pectin esterase of the invention, in the shuttle vector pYES 2.0.

Other strains:

30

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (MAT α ; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

35 *E. coli* strain: DH10B (Life Technologies)

Plasmids:

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

5

pYES 2.0 (Invitrogen)

pA2PE18 (See example 1)

10 **Extraction of total RNA** is performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)⁺RNA is carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.

15

15 **cDNA synthesis:** Double-stranded cDNA is synthesized from 5 µg poly(A)⁺ RNA by the RNase H method (Gubler and Hoffman (1983) Gene 25:263-269, Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY) using the hair-pin modification developed by F. S. Hagen (pers. comm.). The poly(A)⁺ RNA (5 µg in 5 µl of DEPC-treated water) is heated at 70°C for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 µl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM of dATP, dGTP and dTTP and 0.5 mM 5-methyl-dCTP (Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45 µg of oligo(dT)₁₈-Not I primer (Pharmacia) and 1000 units SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA is synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture is gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions.

After the gelfiltration, the hybrids are diluted in 250 μ l second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM MgCl₂, 10 mM (NH₄)₂SO₄, 0.16 mM β NAD⁺) containing 200 μ M of each dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega) and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis is performed by incubating the reaction tube at 16°C for 2 hours and additional 15 min. at 25°C. The reaction is stopped by 10 addition of EDTA to a final concentration of 20 mM followed by phenol and chloroform extractions.

Mung bean nuclease treatment: The double-stranded cDNA is precipitated at -20°C for 12 hours by addition of 2 vols 96% EtOH, 0.2 vol 10 M NH₄Ac, recovered by centrifugation, washed 15 in 70% EtOH, dried and resuspended in 30 μ l Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM ZnSO₄, 0.35 mM DTT, 2% glycerol) containing 25 units Mung bean nuclease (Pharmacia). The single-stranded hair-pin DNA is clipped by 20 incubating the reaction at 30°C for 30 min., followed by addition of 70 μ l 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 5.2 on ice for 30 min.

25 Blunt-ending with T4 DNA polymerase: The double-stranded cDNAs are recovered by centrifugation and blunt-ended in 30 μ l T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating 30 the reaction mixture at 16°C for 1 hour. The reaction is stopped by addition of EDTA to a final concentration of 20 mM, followed by phenol and chloroform extractions, and precipitation for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Adaptor ligation, Not I digestion and size selection:

After the fill-in reaction the cDNAs are recovered by centrifugation, washed in 70% EtOH and dried. The cDNA pellet 5 is resuspended in 25 μ l ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP) containing 2.5 μ g non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 10 ligase (Promega) and incubated at 16°C for 12 hours. The reaction is stopped by heating at 65°C for 20 min. and then 15 cooling on ice for 5 min. The adapted cDNA is digested with Not I restriction enzyme by addition of 20 μ l water, 5 μ l 10x Not I restriction enzyme buffer (New England Biolabs) and 50 units Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C. The reaction is stopped by heating at 65°C 20 for 10 min. The cDNAs are size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated adaptors and small cDNAs. The cDNA is size-selected with a cut-off at 0.7 kb and rescued from the gel by use of β -Agarase 25 (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Construction of libraries: The directional, size-selected cDNA 25 is recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 μ l 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs are desalted by gelfiltration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's 30 instructions. Three test ligations are carried out in 10 μ l ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP) containing 5 μ l double-stranded cDNA (reaction tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0

vector. The ligation reactions are performed by incubation at 16°C for 12 hours, heating at 70°C for 20 min. and addition of 10 µl water to each tube. 1 µl of each ligation mixture is electroporated into 40 µl electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Using the optimal conditions a library is established in *E. coli* consisting of pools. Each pool is made by spreading transformed *E. coli* on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin is added to the plate and the cells were suspended herein. The cell suspension is shaked in a 50 ml tube for 1 hour at 37°C. Plasmid DNA is isolated from the cells according to the manufacturer's instructions using QIAGEN plasmid kit and stored at -20°C.

1 µl aliquots of purified plasmid DNA (100 ng/µl) from individual pools are transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante (1991) Methods Enzymol. 194:182-187) and the transformants are plated on SC agar containing 2% glucose and incubated at 30°C.

Identification of positive clones:

After 3-5 days of incubation, the SC agar plates were replica plated onto a set of SC + galactose agar plates. Those plates were incubated for 2-4 days at 30°C and overlaid with a pectin overlayer gel, containing 1% apple pectin DE 75%, 1% HSB agarose in an appropriate buffer, for detection of pectinolytic activity. After incubation overnight at 30°C, 10-15 ml of a 1% solution of MTAB (mixed alkyltrimethylammonium bromide) was poured onto the overlayer and removed after 1 hour. Pectin methylesterase positive colonies were identified as colonies surrounded by a white halo.

Characterization of positive clones: The positive clones are obtained as single colonies, the cDNA inserts were amplified directly from the yeast colony using biotinylated polylinker primers, purified by magnetic beads (Dynabead M-280, Dynal) system and characterized individually by sequencing the 5'-end of each cDNA clone using the chain-termination method (Sanger et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467) and the Sequenase system (United States Biochemical).
10

Isolation of a cDNA gene for expression in *Aspergillus*:
A pectin esterase-producing yeast colony is inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube is shaken for 2 days at 30°C. The cells are harvested by centrifugation
15 for 10 min. at 3000 rpm.

DNA is isolated according to WO 94/14953 and dissolved in 50 µl water. The DNA is transformed into *E. coli* by standard procedures. Plasmid DNA is isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert is excised using appropriate restriction enzymes and ligated into an *Aspergillus* expression vector.
20

Transformation of *Aspergillus oryzae* or *Aspergillus niger*
Protoplasts may be prepared as described in WO 95/02043, p.
16, line 21 - page 17, line 12, which is hereby incorporated by reference.
25

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM CaCl₂). Protoplasts are mixed with p3SR2 (an *A. nidulans* amds gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 35 4000 (BDH 29576), 10 mM CaCl₂ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the

same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are 5 spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This 10 procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the transformants are inoculated in 10 ml of YPM (cf. 15 below) and propagated. After 2-5 days of incubation at 30°C, the supernatant is removed. The pectin esterase activity is identified by applying 10 µl supernatant to 4 mm diameter holes punched out in an agarose gel containing 1% apple pectin DE 75%, incubation overnight at 30°C and precipitation with 20 MTAB as described above. Pectin esterase activity is then identified by a white halo.

Fed batch fermentation

Fermentations were carried out as fed-batch processes with 25 maltodextrin as carbon source, urea as nitrogen source and yeast extract. The pH was maintained at 7.0 and the temperature was maintained at 34°C during the entire process. After fermentation the pectin esterase was recovered by centrifugation and germ filtration.

30

Purification of the enzyme

The recombinant pectin esterase from *A. oryzae* was purified as follows: A culture supernatant was harvested after 5 days of culture, and centrifuged, germ filtered, and ultrafiltrated on 35 a 3 kDa cut-off Filtron cassette (Minisette) to minimal volume. 25 ml of the ultrafiltrate was dialysed with 50 mM

H_3BO_3 , 5 mM DMG, 1 mM $CaCl_2$, pH 7.0 and applied to a 40 ml Q-Sepharose FF column (Pharmacia, Sweden) equilibrated in the same buffer. After washing the column, bound protein was eluted with a linear NaCl gradient (from 0 to 0.5 M NaCl).

5 Fractions from the column were analysed for pectin esterase activity by the apple pectin assay as described above. Most of the pectin esterase activity was seen in the run-through, whereas most of the protein bound to the column.

10 The pH of the run-through was adjusted to pH 4.5 with CH_3COOH and applied to a 50 ml S-Sepharose HP column (Pharmacia, Sweden) equilibrated in 25 mM $CH_3COOH/NaOH$, pH 4.5. After washing the column, bound protein was eluted with a linear NaCl gradient (from 0 to 0.25 M NaCl). Fractions from the 15 column were analysed for pectin esterase activity. The major part of the pectin esterase activity eluted in one peak, the peak fractions were pooled.

20 Ammonium sulphate (AMS) was added to the pool to a final AMS concentration of 1.6 M and the enzyme was added to a 40 ml Phenyl Toyopearl column equilibrated in 100 mM H_3BO_3 , 10 mM DMG, 2 mM $CaCl_2$, 1.6 M AMS, pH 7.0. After washing the column, bound protein was eluted with a linear AMS gradient (from 1.6 to 0 M AMS). The buffer of the pectin esterase peak was 25 exchanged for 25 mM $CH_3COOH/NaOH$, pH 4.5 by a pass on a 1.4L G25 Sephadex column equilibrated in 25 mM $CH_3COOH/NaOH$, pH 4.5.

30 The pectin esterase enzyme was applied to a 50 ml S-Sepharose HP column equilibrated in 25 mM $CH_3COOH/NaOH$, pH 4.5. After washing the column, bound protein was eluted with a linear NaCl gradient (from 0 to 0.1 M NaCl). Fractions with pectin esterase activity were analysed by SDS-PAGE. The fractions contained one band only.

Electrophoresis

SDS-PAGE electrophoresis was performed in a Mini-Leak 4 electrophoresis unit (Kem-En-Tec, Denmark) as a modified version of the Laemli procedure (Laemmli (1970) *Nature* 227:680-685). Gels were coomassie stained according to the manufacturer's instructions.

Isolation of the DNA sequence shown in SEQ ID No. 1:

The pectin esterase encoding part of the DNA sequence shown in SEQ ID No. 1 coding for the pectin esterase of the invention can be obtained from the deposited organism *Escherichia coli* DSM 10357 by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) *Molecular cloning: A laboratory manual*, Cold Spring Harbor lab., Cold Spring Harbor, NY).

15

Hybridization

Suitable hybridization conditions for determining hybridization between a nucleotide probe and an "analogous" DNA sequence of the invention may be defined as described below. The oligonucleotide probe to be used is the DNA sequence corresponding to the pectin esterase encoding part of the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 4-933 in SEQ ID No. 1.

25 **Hybridization conditions**

The hybridization conditions referred to herein to define an analogous DNA sequence as defined in part b) of the first aspect of the invention which hybridizes to the pectin esterase encoding part of the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 4-933, under at least low stringency conditions, but preferably at medium or high stringency conditions are as described in detail below.

Suitable experimental conditions for determining hybridization at low, medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA

fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 5 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P -dCTP-labeled (specific activity $> 1 \times 10^9$ cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 10 55°C (low stringency), more preferably at least 60°C (medium stringency), still more preferably at least 65°C (medium/high stringency), even more preferably at least 70°C (high stringency), 15 and even more preferably at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

20 **Immunological cross-reactivity:** Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified pectin esterase. More specifically, antiserum against the pectin esterase of the invention may be raised by immunizing rabbits (or other rodents) according to 25 the procedure described by N. Axelsen et al. in: *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, 1982 (more specifically p. 27-31). Purified 30 immunoglobulins may be obtained from the antisera, for example by salt precipitation $((\text{NH}_4)_2\text{SO}_4$), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunological characterization of proteins may be done either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: 35 *Handbook of Experimental Immunology* (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by

crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

5 **Media**

YPD: 10 g yeast extract, 20 g peptone, H₂O to 900 ml.

Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone, H₂O to 900 ml.

10 Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H₂O ad 1000 ml, sterile filtered.

15 SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H₂O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

20 SC-agar: SC-URA, 20 g/l agar added.

PEG 4000 (polyethylene glycol, molecular weight = 4,000) (BDH, England)

25 1% apple pectin DE 75% (Herbstreith, Germany)

1% HSB agarose (FMC Litex A/S, Denmark)

MTAB (mixed alkyltrimethylammonium bromide) (Sigma)

30

EXAMPLES

EXAMPLE 1

35

Cloning and expression of a pectin esterase from *Meripilus*

giganteus CBS 521.95

mRNA was isolated from *Meripilus giganteus*, CBS No. 521.95, grown in cellulose-containing fermentation medium with 5 agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *M. giganteus*, CBS No. 521.95, consisting of approx. 10⁶ individual clones was constructed in *E. coli* as described with a vector background 10 of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

15 Pectin esterase-positive colonies were identified and isolated on SC-agar plates with the apple pectin assay. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequence of the cDNA encoding the pectin esterase is shown in SEQ ID No. 1 and the corresponding 20 amino acid sequence is shown in SEQ ID No. 2. In SEQ ID No. 1 DNA nucleotides from No 4 to No. 933 define the pectin esterase encoding region.

25 The cDNA is obtainable from the plasmid in DSM 10357.

30 Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the pectin esterase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the pectin esterase gene was purified. The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2PE18.

35 After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme activity as described above. Some of the transformants had pectin esterase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the pectin esterase in *Aspergillus oryzae*.

10 EXAMPLE 2**Homology to published pectin esterases**

A homology search with the pectin esterase of the invention 15 against nucleotide and protein databases was performed. The homology search showed that the most related pectin esterases was a pectin esterase from *Aspergillus aculeatus* and a pectin esterase from *Aspergillus tubigensis* (previously said to be from *A. niger*).

According to the method described in the "DETAILED DESCRIPTION 20 OF THE INVENTION" the DNA homology of the pectin esterase of the invention against most prior art pectin esterases was determined using the computer program GAP. The pectin esterase of the invention has only 54% DNA homology to the pectin esterase from *Aspergillus aculeatus* (WO 94/25575) and the pectin esterase of the invention has only 56% DNA homology to the pectin esterase from *Aspergillus tubigensis* (Khanh et al. 25 (1990) "Nucleotide and derived amino acid sequence of a pectinesterase cDNA isolated from *Aspergillus niger* strain RH5344", Nucleic Acids Res. 18:4262; Khanh et al. (1991) "Characterization and expression of a genomic pectin methyl esterase-encoding gene in *Aspergillus niger*", Gene 106:71-77).

35 According to the method described in the "DETAILED DESCRIPTION OF THE INVENTION" the polypeptide homology of the pectin esterase of the invention against most prior art pectin

esterases was determined using the computer program GAP. The pectin esterase of the invention has only 47% polypeptide homology to the pectin esterase from *Aspergillus aculeatus* and the pectin esterase of the invention has only 44% polypeptide homology to the pectin esterase from *Aspergillus tubigensis*

5 This shows that the pectin esterase of the invention indeed is distant from any known pectin esterases.

10

EXAMPLE 3

15 **Purification and characterization of the recombinant pectin esterase of the invention**

The pectin esterase was produced by fed batch fermentation of *A. oryzae* expressing the enzyme as described in Materials and Methods above.

20 The recombinant pectin esterase was purified and characterized by the methods described in the Materials and Methods section above. The molecular weight of the enzyme was determined to 37 kDa by SDS-PAGE.

25

EXAMPLE 4

30 **In situ gelation of strawberry jam**

Free methanol as an indication of the gelling properties

Materials and methods:

Pectinesterase batch PPJ 4300, 423 PEU/g

Pectinesterase: *Meripulus giganteus*, 2,4 PEU/g

35 Strawberries, frozen, sort : Senga sengana

The activity of the pectinesterase is given in Pectin-Esterase-Units (PEU) defined as the amount of enzyme which under standardised conditions liberates 1 mmol carboxyl groups per minute (Novo Nordisk assay ABT-SM-0005.02.1, Available 5 upon request from Novo Nordisk A/S).

Jam preparation

The preparation of the jam is carried out according to the standard procedure for jam preparation (Novo Nordisk Standard 10 Operation Procedure, ABF-SP-4002.02/01, Available upon request from Novo Nordisk A/S).

A 300 g portion of jam was prepared. The ratio of strawberry and sugar was: 180g berries + 120 g sugar. After an initial 15 pre-cooking the fruit slurry was divided into 2 x 3 - 50 g portions, the samples were cooled to 40°C and enzyme was added as follows:

- 1) control with no enzyme added
- 20 2) PE, PPJ 4300, 10 PEU/kg fruit
- 3) PE, *Meripulus giganteus*, 10 PEU/kg fruit

The samples were sealed and allowed to stand for 1 hour, then 25 the samples were heat treated for 3 min at 90°C in order to inactivate the enzyme.

In the present trials the quantification of free methanol was carried out at Alfred Jørgensens Lab. A/S, Copenhagen. The samples were initially distilled, then analysed using a 30 capillary gaschromatograph in combination with mass spectrometry detection.

Results and discussion:

The results appears from Table 1 below:

Table 1, Methanol formation

	Methanol, ppm
Control	12 ppm
PE, PPJ 4300, 10 PEU/kg fruit	200 ppm
PE, <i>M.gig.</i> , 10 PEU/kg fruit	340 ppm

5 It is clearly demonstrated that pectinesterase from *Meripulus giganteus*, resulting in 340 ppm free methanol, is superior to the more traditional *Aspergillus* derived PE, resulting in a free methanol formation of 200 ppm.

10 If the pectin content of strawberries is estimated to 0.6 % and the degree of esterification (DE) is estimated to 70%, it can be calculated, that the final DE of the traditional PE product results in a DE of 52, while *Meripulus giganteus* - PE results in a DE of 38.

15 The degree of esterification is of importance for the textural characteristics of *in situ* gelated jam and also for other PE modified vegetable products. The degree of esterification determines the number of Ca-bridges that can be formed and is thus related to the gel strength or the viscosity of a certain product.

20

25 It is thus obvious that the *Meripulus giganteus* derived enzyme is a useful tool in creating strong and useful gels. The enzyme is probably also as an even stronger alternative to the chemical modification of extracted pectins, than the traditional *Aspergillus* derived PE product.

SEQUENCE LISTING

SEQ ID No. 1 shows the DNA sequence of the full-length cDNA sequence comprised in the DNA construct transformed into the deposited Escherichia coli DSM 10357.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1128 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Meripilus giganteus
- (B) STRAIN: CBS 521.95

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 4..933

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGA ATG CAG TCC CAC TTG TTC TTG CTG TTC TCT TAC CTC ATC GCC TGT
Met Gln Ser His Leu Phe Leu Leu Phe Ser Tyr Leu Ile Ala Cys
1 5 10 15

48

GCG TCT GCG CTC AGC AGT CCG CCT GCA GGC GCA ATC ACC GTC GGC TCC
Ala Ser Ala Leu Ser Ser Pro Pro Ala Gly Ala Ile Thr Val Gly Ser
20 25 30

96

GGT GGC AAG TAT TCA ACG TTG TCC GCA GCC CTC AAG GAC ACG TCG AGC

144

Gly Gly Lys Tyr Ser Thr Leu Ser Ala Ala Leu Lys Asp Thr Ser Ser
 35 40 45

TCC GTC TAC TTC GTG TTC TCC GGG ACA TAC ACG GAC ACC GCG ATT ATT
 Ser Val Tyr Phe Val Phe Ser Gly Thr Tyr Thr Asp Thr Ala Ile Ile
 50 55 60

ACT CCG CCG AAC GTG AAG GTG TAC GGG CAG ACG AAC ACA CCG TCG TCC
 Thr Arg Pro Asn Val Lys Val Tyr Gly Gln Thr Asn Thr Pro Ser Ser
 65 70 75

TAT ACC GGC AAT ACT GTC ACC ATC ACC AAT AAC ATT CCG GCC TCC AAG
 Tyr Thr Gly Asn Thr Val Thr Ile Thr Asn Asn Ile Pro Ala Ser Lys
 80 85 90 95

GCG GGC TCC AAT GAT GCG AGC GGG ACG GTG CAA GTG CAC GCC GAG AAC
 Ala Gly Ser Asn Asp Ala Ser Gly Thr Val Gln Val His Ala Glu Asn
 100 105 110

GTG TCA CTC TAC AAC CTA AAC ATC GCG AAC ACG TAC GGC AAG ACA GTC
 Val Ser Leu Tyr Asn Leu Asn Ile Ala Asn Thr Tyr Gly Lys Thr Val
 115 120 125

GAC CAG GCG CAA GCG ATC GCA CTG AGC GTG CAG GCG GGC CAG TTC GGC
 Asp Gln Ala Gln Ala Ile Ala Leu Ser Val Gln Ala Gly Gln Phe Gly
 130 135 140

GCA TAC GGA CTC AAG ATT ACG GGG GAC CAG GAC ACC CTC CTA GCT AAC
 Ala Tyr Gly Leu Lys Ile Thr Gly Asp Gln Asp Thr Leu Leu Ala Asn
 145 150 155

GTC GGC GCG CAA TAC TAT GCG AAC AGC TGG ATA GAA GGC GCT GTG GAC
 Val Gly Ala Gln Tyr Tyr Ala Asn Ser Trp Ile Glu Gly Ala Val Asp
 160 165 170 175

TTC ATA TTC GGG ATG CAA GCC TCG ATC TGG ATC ACC CGC TCG GTC ATC
 Phe Ile Phe Gly Met Gln Ala Ser Ile Trp Ile Thr Arg Ser Val Ile
 180 185 190

AAC ACG ATC GGG AGC GGG TGC ATC ACC GCG TCG GGG CGC TCG AGT AAC
 Asn Thr Ile Gly Ser Gly Cys Ile Thr Ala Ser Gly Arg Ser Ser Asn
 195 200 205

GAC GGC TTC TGG TAT GTC ATC GAC AGC TCG ACC GTG CAG GGC ACC GGG

Asp Gly Phe Trp Tyr Val Ile Asp Ser Ser Thr Val Gln Gly Thr Gly			
210	215	220	
ACG GCG TAC CTC GGG CGC CCC TGG CGC GAC TAC GCG CGC GTC GTG TTC			720
Thr Ala Tyr Leu Gly Arg Pro Trp Arg Asp Tyr Ala Arg Val Val Phe			
225	230	235	
CAG AAG AGC ACG CTC GGG ACC AAC GTG CCC GCG GCC GGC TGG TCG ATT			768
Gln Lys Ser Thr Leu Gly Ser Asn Val Pro Ala Ala Gly Trp Ser Ile			
240	245	250	255
TGG AAC GTC GGC ACC CCG CAG ACC GAC CAT GTC ACG TTC GCC GAG TAC			816
Trp Asn Val Gly Thr Pro Gln Thr Asp His Val Thr Phe Ala Glu Tyr			
260	265	270	
GGG AAC ACC GGC CCC GGC GCG TCC GGC ACG CGC GCG AGC TTC AGC ACG			864
Gly Asn Thr Gly Pro Gly Ala Ser Gly Thr Arg Ala Ser Phe Ser Thr			
275	280	285	
AAG CTG TCC GCG CCG GTC GCG ATC AAG ACG GTG CTG AAC TCG ACG AGC			912
Lys Leu Ser Ala Pro Val Ala Ile Lys Thr Val Leu Asn Ser Thr Ser			
290	295	300	
TGG ATC GAC CCC GCC TTC CTC TGACCCCGCC GACGGCAACG ACAAAACGAGA			963
Trp Ile Asp Pro Ala Phe Leu			
305	310		
GAGAGCGAGG GCAGCAGAGG GACGAGTGCC GAGTGCCGGT GCGGCTGAGG GCTATGACGT			1023
CGATCGATGG TCGGCTTGAC GTCGTTGTAC ACGTACGGAT GCAGTCATCG AACGTTCGGT			1083
TGCGACTTGC CAAAAAAA AAAAAAAA AAAAAAAA AAAAAA			1128

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 310 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Gln Ser His Leu Phe Leu Leu Phe Ser Tyr Leu Ile Ala Cys Ala
 1 5 10 15

Ser Ala Leu Ser Ser Pro Pro Ala Gly Ala Ile Thr Val Gly Ser Gly
 20 25 30

Gly Lys Tyr Ser Thr Leu Ser Ala Ala Leu Lys Asp Thr Ser Ser Ser
 35 40 45

Val Tyr Phe Val Phe Ser Gly Thr Tyr Thr Asp Thr Ala Ile Ile Thr
 50 55 60

Arg Pro Asn Val Lys Val Tyr Gly Gln Thr Asn Thr Pro Ser Ser Tyr
 65 70 75 80

Thr Gly Asn Thr Val Thr Ile Thr Asn Asn Ile Pro Ala Ser Lys Ala
 85 90 95

Gly Ser Asn Asp Ala Ser Gly Thr Val Gln Val His Ala Glu Asn Val
 100 105 110

Ser Leu Tyr Asn Leu Asn Ile Ala Asn Thr Tyr Gly Lys Thr Val Asp
 115 120 125

Gln Ala Gln Ala Ile Ala Leu Ser Val Gln Ala Gly Gln Phe Gly Ala
 130 135 140

Tyr Gly Leu Lys Ile Thr Gly Asp Gln Asp Thr Leu Leu Ala Asn Val
 145 150 155 160

Gly Ala Gln Tyr Tyr Ala Asn Ser Trp Ile Glu Gly Ala Val Asp Phe
 165 170 175

Ile Phe Gly Met Gln Ala Ser Ile Trp Ile Thr Arg Ser Val Ile Asn
 180 185 190

Thr Ile Gly Ser Gly Cys Ile Thr Ala Ser Gly Arg Ser Ser Asn Asp
 195 200 205

Gly Phe Trp Tyr Val Ile Asp Ser Ser Thr Val Gln Gly Thr Gly Thr
 210 215 220

Ala Tyr Leu Gly Arg Pro Trp Arg Asp Tyr Ala Arg Val Val Phe Gln
225 230 235 240

Lys Ser Thr Leu Gly Ser Asn Val Pro Ala Ala Gly Trp Ser Ile Trp
245 250 255

Asn Val Gly Thr Pro Gln Thr Asp His Val Thr Phe Ala Glu Tyr Gly
260 265 270

Asn Thr Gly Pro Gly Ala Ser Gly Thr Arg Ala Ser Phe Ser Thr Lys
275 280 285

Leu Ser Ala Pro Val Ala Ile Lys Thr Val Leu Asn Ser Thr Ser Trp
290 295 300

Ile Asp Pro Ala Phe Leu
305 310

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 11 _____, lines 6 - 18 _____

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country)

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit 4 July, 1995

Accession Number CBS 521.95

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 11 _____, lines 19 - 30 _____.

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit 6 December, 1995

Accession Number DSM.10357

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

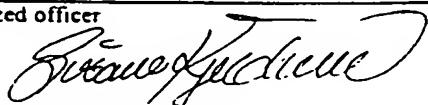
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CLAIMS

1. A DNA construct comprising a DNA sequence encoding an enzyme exhibiting pectin esterase activity, which DNA sequence comprises
 - a) the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *E. coli* DSM 10357, or
 - b) an analogue of the DNA sequence defined in a) which
 - i) is at least 60% homologous with the DNA sequence defined in a), or
 - ii) hybridizes with the DNA sequence shown in positions 4-933 in SEQ ID NO 1 at low stringency, or
 - iii) encodes a polypeptide which is at least 50% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence defined in a), or
 - iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified pectin esterase encoded by the DNA sequence defined in a).
 2. The DNA construct according to claim 1, in which the DNA sequence encoding an enzyme exhibiting pectin esterase activity is obtainable from a microorganism, preferably a filamentous fungus, a yeast, or a bacteria.
 3. The DNA construct according to claims 2, in which the DNA sequence is obtainable from a strain of the *Basidiomycota*, preferably a strain of the class of *Hymenomycetes*, more

preferably a strain of the order *Aphyllophorales*, more preferably a strain of the *Polyporaceae* family, such as the genera *Meripilus*, in particular a strain of *Meripilus giganteus*.

5

4. The DNA construct according to claim 3, in which the DNA sequence is isolated from or produced on the basis of a DNA library of the strain *Meripilus giganteus* CBS 521.95.

10 5. The DNA construct according to claim 2, in which the DNA sequence is obtainable from a strain of *Aspergillus*, *Saccharomyces*, *Bacteroides*, *Erwinia*, *Pseudomonas* or *Clostridium*.

15 6. The DNA construct according to claim 1, in which the DNA sequence is isolated from *Escherichia coli* DSM 10357.

7. A recombinant expression vector comprising a DNA construct according to any of claims 1-6.

20 8. A cell comprising a DNA construct according to any of claims 1-6 or a recombinant expression vector according to claim 7.

25 9. The cell according to claim 8, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

30 10. The cell according to claim 9, which is a strain of *Fusarium* or *Aspergillus* or *Trichoderma*, in particular a strain of *Fusarium graminearum*, *Fusarium cerealis*, *Aspergillus niger*, *Aspergillus Oryzae*, *Trichoderma harzianum* or *Trichoderma reesei*.

35 11. The cell according to claim 9, which is a strain of *Meripilus* sp., in particular *Meripilus giganteus*.

12. The cell according to claim 11, being the strain *Meripilus giganteus* CBS No. 521.95.

5 13. The cell according to claim 9, which is a strain of *Saccharomyces*, in particular a strain of *Saccharomyces cerevisiae*.

10 14. A method of producing an enzyme exhibiting pectin esterase activity, the method comprising culturing a cell according to any of claims 8-13 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

15 15. An enzyme exhibiting pectin esterase activity, which

20 (a) is encoded by a DNA construct according to any of claims 1-6, or the recombinant expression vector according to claim 7, or

25 (b) produced by the method according to claim 14, and/or

25 (c) is immunologically reactive with an antibody raised against a purified pectin esterase encoded by the pectin esterase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *E. coli* DSM 10357.

30 16. The enzyme according to claim 15, which has the amino acid sequence SEQ ID NO 2.

17. A composition comprising the enzyme according to claim 15 or 16.

35 18. The enzyme composition which is enriched in an enzyme exhibiting pectin esterase activity according to claim 15 or 16.

19. The composition according to claim 18, which additionally comprises an α -arabinosidase, β -galactosidase, α -glucuronidase, β -xylosidase, xylan acetyl esterase, xylanase, arabinase, rhamnogalacturonase, rhamnogalacturonan acetylesterase, pectin acetylesterase, galactanase, polygalacturonase, pectin lyase, pectate lyase, or pectin methylesterase.

5 20. Use of an enzyme according to claim 15 or 16 or an enzyme composition according to any of claims 17 to 19 for improving the firmness of a pectin-containing material.

10 21. Use of an enzyme according to claim 15 or 16 or an enzyme composition according to any of claims 17 to 19 for increasing the viscosity of a pectin-containing material.

15 22. The use according to claim 20 and 21, in which the pectin-containing material is a fruit or vegetable material.

20 23. Use of an enzyme according to claim 15 or 16 or an enzyme composition according to any of claims 17 to 19 for demethylation of pectin.

25 24. Use of an enzyme according to claim 15 or 16 or an enzyme composition according to any of claims 17 to 19 in the preparation of feed.

30 25. Use of an enzyme according to claim 15 or 16 in combination with a polygalacturonase containing enzyme preparation for reducing the viscosity of a plant cell wall derived material.

35 26. Use of an enzyme according to any of claim 15 or 16 or an enzyme composition according to any of claims 17 to 19 in the production of wine or juice.

27. An isolated substantially pure biological culture of the deposited strain *Escherichia coli* DSM 10357.

INTERNATIONAL SEARCH REPORT

1

International application No.

PCT/DK 97/00073

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0388593 A1 (RÖHM GMBH CHEMISCHE FABRIK KIRSCHENALLE), 26 Sept 1990 (26.09.90) --	1-27
X	EMBL, Databas Genbank/DDBJ, Anpecase, accession no. X52902, Khan N.Q. et al: "Nucleotide and derived amino acid sequence of a pectinesterase cDNA isolated from Aspergillus niger strain RH 5344", Nucleic Acids Res. 18:4262-4262 (1990) 1990-07-25 --	1-27

 Further documents are listed in the continuation of Box C. See patent family annex.

- Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

15 May 1997

Date of mailing of the international search report

11-06-1997

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INTERNATIONAL SEARCH-REPORT

2

International application No.
PCT/DK 97/00073

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 5, BIOSIS, Dialog accession no. 2458386, Biosis accession no. 66005291, Yoshihara O. et al: "Purification and Properties of Acid Pectin Esterase EC-3.1.1.11 from <i>Corticium-RolfsII</i> ", Agric Biol Chem 41 (12). 1977 (RECD 1978) 2335-2342 --	1-27
A	Dialog Information Services, File 5, BIOSIS, Dialog accession no. 10020821, Biosis accession no. 95020821, Frittrang A K et al: Characterization and Partial Purification of Pectinesterase a Differentiation-Specific Enzyme of <i>Uromyces-Viciae-</i> <i>Fabae</i> ", J Gen Microbiol 138 (10). 1992. 2213-2218 --	1-27
A	Chemical Abstracts, Volume 104, No 23, 9 June 1986 (09.06.86), (Columbus, Ohio, USA), Dudchenko, L.G., "Effect of metal ions on the activity of pectolytic enzymes in the higher basidiomycete <i>Coriolus hirsutus</i> (Fr) Quel.", page 353, THE ABSTRACT No 202881, Dopov.Akad.Nauk Ukr. RSR, Ser.B:Geol.... 1986, 2, 57-61 --	1-27
A	WO 9425575 A1 (NOVO NORDISK A/S), 10 November 1994 (10.11.94) --	1-27
A	Biologia (Bratislava), Volume 44, No 12, 1989, Oskar Markovic et al, "Purification and Characterization of an <i>Aspergillus Niger</i> Pectinesterase" page 1185 - page 1189 --	1-27
A	US 4200694 A (SHIGETAKA ISHII ET AL), 29 April 1980 (29.04.80) -----	1-27

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/04/97

International application No. PCT/DK 97/00073	
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WO 9425575 A1	10/11/94	AU 6676794 A EP 0696319 A	21/11/94 14/02/96
US 4200694 A	29/04/80	DE 2843351 A,B,C FR 2405263 A,B JP 1147089 C JP 54055784 A JP 57024755 B JP 1132369 C JP 54055792 A JP 57018796 B	12/04/79 04/05/79 12/05/83 04/05/79 26/05/82 27/01/83 04/05/79 19/04/82

